# INVESTIGATING STERIC PROTECTION OF DNA IN THE PRESENCE OF NULCEASES

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# INVESTIGATING STERIC PROTECTION OF DNA IN THE PRESENCE OF NUCLEASES

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# ABBREVIATIONS

| | |
|---|---|
| μM | Micro molar |
| μm | Micrometer |
| μL | Micro litre |
| BP | Base Pair |
| B14 | ssDNA target containing 14 bases with no polymeric tail |
| PEG | Polyethylene glycol |
| dT | Thymine |
| LNA | Locked nucleic acid |
| DNA | Deoxyribonucleic acid |
| FITC | Fluorescein isothiocyanate |
| RT | Room temperature |
| Oligos | Oligonucleotides |
| *DNase I* | Enzyme that specifically cleaves DNA linkages |
| Coupling | Formation of a covalent bond between the probe and microsphere |
| Probe | ssDNA coupled to polystyrene microsphere |
| Primary Target | ssDNA that binds with the probe to form a duplex |
| Primary Hybridization | Formation of a duplex between the probe and target |
| PBS 0.02% Tween | Phosphate buffer solution with 0.02% Tween |
| Tris EDTA | Ethylene diamine tetra acetic acid |

# ABSTRACT

In the human body, DNA-linked colloidal assemblies are prone to cleavage by nucleases, yielding the uncontrolled release of particles and any associated therapeutics. Thus, for *in vivo* applications, the DNA-linkages must be protected from cleavage by serum nucleases. The goal of this research is to stabilize DNA duplexes in the presence of nucleases by chemically modifying the primary target. The effects of sterically protecting DNA duplexes from nuclease activity by including a polymeric “tail” on oligonucleotide targets and by including LNA bases in the target sequence were investigated. The variables explored included the effect of tail chemistry as well as tail length on the kinetics and extent of nuclease activity. In DNA digests, two types of polymeric "tails" were compared: polyethylene glycol (PEG) chains and single stranded thymine-based strands (dT). Long and short PEG and thymine tails of equivalent lengths were compared. Ezymatic digests were also performed on fluorescently labeled primary targets modified with oligonucleotide analogs called LNA, locked nucleic acids. Flow cytometry was used to quantify the hybridization activity and measure the probe-target duplex density as well as to determine time-dependence of nuclease activity by monitoring the number of duplexes remaining following incubation with *DNase I*. Significant clipping was observed for all DNA targets tested and indicated that various polymeric tails did not significantly hinder nuclease activity. These results indicate that the relatively short polymeric tail lengths do not have appreciable effects on the hindrance of nuclease activity. LNA digests, on the other hand, showed enhanced stability of primary duplexes in the presence of nucleases after 24 hours and suggested that LNA may be used as an alternative to DNA to stabilize colloidal assemblies for drug delivery.

# CHAPTER 1

# INTRODUCTION

## Research Objectives

DNA is promising as an assembly tool for cancer drug delivery vehicles. The Milam lab is currently investigating whether colloidal assemblies bound by DNA linkages can potentially serve as drug delivery vehicles. There is one key limitation to this idea since, in the human body, DNA-linked colloidal assemblies may be prone to cleavage by nucleases, yielding the uncontrolled release of particles and any associated therapeutics. Thus, for *in vivo* applications, the DNA-linkages must be protected from cleavage by serum nucleases.

The purpose of this research is to stabilize DNA duplexes in the presence of nucleases. We added polymeric tails to the primary targets in order to sterically hinder nuclease activity. We explored optimizing the length and chemistry of polymeric "tails" on each primary target. The variables explored included the effects of tail chemistry and tail length on the kinetics and extent of nuclease activity. We expected the addition of polymeric tails to the end of each DNA target would stabilize the DNA linkages in the presence of nucleases.

Hybridization of ssDNA probes with ssDNA targets bridged particles together in the colloidal assemblies. In our experiments, single stranded 20 base long DNA is coupled as an immobilized strand to a 1 μm polystyrene bead. Next a primary target strand is hybridized to the immobilized DNA. A duplex forms between the ssDNA immobilized to

the bead surface and the primary target. This hybridization event binds the colloidal assembly that will serve as a drug delivery vehicle.

In this work, my experiments explored the minimum tail length needed to maintain duplex stability in the presence of nucleases. I also explored using locked nucleic acids (LNA) as an alternative to DNA. As a nucleic acid analogue, LNA has been reported to possess superior nuclease resistance compared to DNA and may thus provide the chemical stability lacking in our current experimental system. The effects of sterically protecting DNA duplexes from nuclease activity by including a polymeric "tail" on oligonucleotide targets was investigated.

**Literature Review**

James Storhoff and Chad Mirkin argue that DNA is “one of the most programmable ‘assemblers’ available to the synthetic chemist and materials scientist” [1]. The fledgling development of using DNA as a biological assembly tool has demonstrated that DNA is useful in developing nanostructured materials, examining electronic interactions between particles and creating new detection platforms [1]. The superb molecular recognition properties of DNA were first investigated by Seeman and co-workers. They created “branched junctions” of DNA duplexes with the intent to serve as smaller components that would later be assembled in to large 2- and 3-D structures [1].

The Milam lab seeks to take advantage of the programmable assembly properties of DNA to ultimately form colloidal assemblies that will serve as drug delivery vehicles.

Biomacromolecules are attractive for therapeutic applications as they may be functionalized to specifically target receptors on cells as illustrated in Figure 1. One key challenge to this approach, however, is avoiding recognition and clearance by macrophages. To date, polyethylene glycol (PEG) has commonly served as protective coatings material for drug delivery vehicles. My project involved optimizing the length and density of PEG–terminated oligonucleotides that serve to form a protective coating around the assemblies.

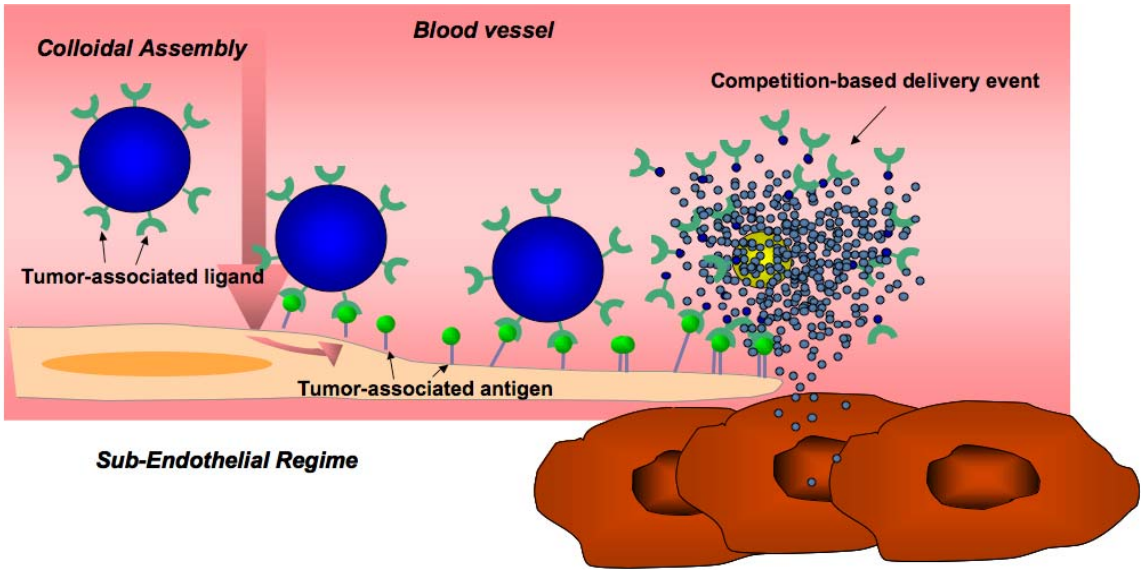


**Figure 1.** Schematic illustrating the long-term goal of delivering and then releasing particles at targeted diseased tissue. DNA hybridization events mediate the assembly and then the disassembly of the particles.

DNA may also be used as a disassembly tool. Several methods have been reported for dissociating surface-bound DNA duplexes. Dissociation may be achieved via thermal melting and enzymatic digest to cleave oligonucleotides. Jin et al., for example, reported thermal melting of DNA linkages on gold nanoparticles as a function of particle size,

density of duplexes on the nanoparticle surface, salt concentration, and distance between particles [2].

Thermal dissociation is not suitable for *in-vivo* conditions, as they require the surrounding environment to be altered. In previous studies, Seferos et al. have examined the stability of DNA with respect to nucleases [4]. They report that the stability of immobilized DNA on nanoparticles is enhanced [4]. They also attribute the reduction of nuclease activity to an increase in the local salt concentration surrounding the nanoparticle [4]. Though this research is promising in stabilizing polyvalent nanoparticles *in vitro*, it fails to address the stabilizing of DNA duplexes *in vivo*.

The Milam lab uses competitive hybridization events as an alternative to thermal melting or enzymatic cleavage [6]. Competitive hybridization occurs if a secondary DNA target is introduced to particles and replaces the previous DNA target. This competitive strand binds to an immobilized probe displacing the surface-bound DNA duplexes and causing the two nanoparticles to dissociate.

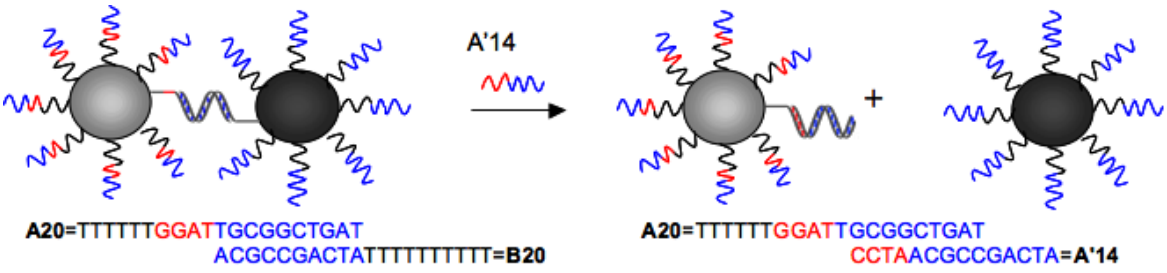


**Figure 2.** Schematic illustrating the assembly and disassembly of polystyrene microspheres via competitive hybridization. Here, two individual microsopheres are initially linked via hybridization of ssDNA immobilized on the surface of each particle.

The particles redisperse due to a competitive hybridization event in which secondary, longer DNA (A'14) targets are introduced.

For *in vivo* applications, the stability of the DNA duplexes that bind the colloidal assemblies is a primary concern. These delicate DNA-mediated colloidal assemblies are prone to cleavage by nucleases when introduced to the body. Uncontrolled cleavage may result in the uncontrolled release of particles and any associated therapeutics. Thus, ultimately for *in vivo* applications, the DNA-linkages must be protected from cleavage by serum nucleases. To increase the stability of DNA linkages for *in vivo* applications, we propose adding polymeric tails to existing DNA linkages. By functionalizing the target strands of DNA with polymeric tails, we hope to create an enzyme resistant brush that will provide steric hindrance to cleavage by nucleases. This will add stability to the colloidal assemblies when used as drug carriers in the human body. PEG functionalized DNA yields several advantages as it does not inhibit initial assembly, provides protection from nuclease activity, and allows assemblies to be manufactured at room temperature reducing costs [3]. Optimizing the length and chemistry of polymeric "tails" on one end of each target DNA strand is crucial to sterically hinder nucleases from finding and clipping the DNA duplex segments.

Locked nucleic acids (LNA) may be a viable alternative to DNA to maintain duplex stability in the presence of nucleases. LNA has been reported to possess superior nuclease resistance compared to DNA and may thus provide the chemical stability lacking in our current experimental system [8]. LNA is non toxic and exhibits high stability and affinity.

LNA has been shown to be non toxic in the body and may be suitable for *in vivo* applications [8]. They also have shown stability in serum and exhibit a high binding affinity to complementary DNA sequences [8].

The effects of sterically protecting DNA duplexes from nuclease activity by including a polymeric "tail" on oligonucleotide targets and integrating LNA in target strands were investigated. Results from the studies reported here may provide key insights into translating the current model *in vivo* system into a practical drug delivery vehicle for individualized delivery.

# CHAPTER 2

# EXPERIMENTAL PROCEDURES

## Materials

For the coupling, nonfluorescent 1.10 μm carboxylated polystyrene microspheres were obtained from Bangs Laboratories, Inc (Bangs Laboratories, Fishers, IN). These carboxylated beads were used as the substrate for coupling A20 ssDNA probes which were obtained from Invitrogen. The FITC-labeled primary targets for hybridization were also obtained from Invitrogen (Carlsbad, CA). The restriction enzyme, *DNase I*, and the corresponding NE Buffer for *DNase I* that were used in all digest kinetics studies were purchased from New England Biolabs.

The candidate DNA and LNA probesand targets are given in Table 1 below. All DNA targets have a 14 base-long hybridization segment (underlined) with the probe sequence (not shown), which is immobilized on a 1.10 μm polystyrene microsphere surface.

**Table 1**: Partial list of DNA sequences and corresponding "tail" chemical composition for select target sequences. Each target is labeled on the 5' end with the fluorescent molecule carboxyfluorescein (FAM) and the hybridization segment (with the complementary probe) is underlined. The tail segment is shown in bold.

| Function | Nomenclature | Sequence and Tail Chemistry |
|---|---|---|
| Immobilized DNA probe | A20 | 5'-TTTTTTGGATTGCGGCTGAT-3' |
| Immobilized LNA probe | L20 | 5'-AmMC6-TTTTTT$^{L}$GGA$^{L}$TTG$^{L}$CGG$^{L}$CTG$^{L}$AT-3' |
| Soluble, noncomplementary target | NC20 | 5'-TTTTTTGGATTGCGGCGTGAT-3' |
| Soluble, complementary DNA target | B14 | 5'-FAM-ATCAGCCGCAATCC-3' |
| | dT5 | 5'-FAM-**TTT TT**A TCA GCC GCA ATC C-3' |
| | dT3 | 5'-FAM-**TTT** ATC AGC CGC AAT CC -3' |
| | 6PEG | 5'-FAM-**$(CH_2CH_2O)_6$**-ATC AGC CGC AAT CC -3' |
| | 3PEG | 5'-FAM-**$(CH_2CH_2O)_3$**-ATC AGC CGC AAT CC -3' |
| | 30PEG | 5'-FAM- ATC AGC CC-3' |
| | Ultralong Triblock | 5'-FAM-ATC AGC CGC AAT CC-**$(dT)_{86}$-$(CH_2CH_2O)_{35}$**-3' |
| Soluble, compelementary LNA target | B9 ET LNA | 5'-FAM-A$^{L}$TCA$^{L}$GCC$^{L}$GC-3' |

**Particle Preparation: Coupling of Oligonucleotides to Particle Surface**

Single stranded DNA probes, A20, were covalently coupled to 1 µm polystyrene microspheres using the following procedure: the beads were aliquoted from the stock suspension and 100 µL of the coupling buffer was added to 10 µL of the 10% beads to make an ~1% suspension. This solution was vortexed, sonicated, and centrifuged and then washed in the coupling buffer. After being re-suspended in the 125 µL of the coupling buffer, a 10 µM solution of A20 DNA and Tris EDTA (pH 7.4) was added to the suspension. This 1% bead suspension was then incubated on the roto-mixer for 2 hours before being washed and finally resuspended in 100 µL of PBS and 0.02% Tween.

**Hybridization of Primary Targets without Polymeric Tails**

We first examined the hybridization of DNA of various base lengths in order to determine which target candidates resulted in maximum hybridization activity with A20-

functionalized microspheres. We examined the hybridization of DNA target strands with lengths of 8, 10, 12, 14, and 16 base pairs.

After the polystyrene beads were functionalized with probe strands, we took 2.5 µL of the previously coupled 1% bead suspension and washed them in 100 µL PBS and 0.02% Tween. A 10 µM solution of FITC-labeled DNA targets and Tris EDTA (pH 8.0) was then added to the beads in order to promote formation of fluorescently-labeled duplexes. This mixture of FITC-labeled DNA and coupled beads was incubated at room temperature on the roto-mixer for 4 hours and then washed and re-suspended in 100 µL PBS 0.02% Tween. Flow cytometry was used to quantify the density of labeled duplexes on the bead population.

**Hybridization of Primary Targets with Polymeric Tails**

We next investigated including polymeric tails on the primary targets affected hybridization activity. Targets possessing PEG or Thymine (dT) tails of various molecular weights were incubated with 1% 1 µm A20 probe-functionalized beads. Each target strand of DNA was FITC labeled and again flow cytometry was using to quantify the density of labeled duplexes resulting from hybridization events.

To hybridize primary targets with polymeric tails, 3.0 µL of 100% solution of 1.0 µm A20-functionalized polystyrene microspheres was suspended in 100 µL of PBS with 0.02% Tween. In a separate tube, 20 µL of FAM-labeled DNA was diluted with 180 µL Tris EDTA (pH 8.0) to a 10 µM solution. Next 100 µL of the 10 µM diluted DNA

solution was added to the 1% bead suspension and incubated at room temperature for 24 hours on the rota-mixer. The suspensions were then washed 3X in 100 µL PBS with 0.02% Tween with centrifuge times of 2 minutes at 9.9 kG. The samples were resuspended in 100 µL PBS with 0.02% Tween.

### *DNase I* Digest Kinetics of DNA Target Sequences with Polymeric Tails

The next set of experiments involved incubating the prepared suspensions with *DNase I*, a nuclease that specifically cleaves DNA strands. The time-dependent nuclease activity was monitored using flow cytometry to determine the number of labeled duplexes remaining following incubation with *DNase I*.

These experiments started with probe-functionalized 1.10 µm beads that have been hybridized with FITC-labeled target strands with and without various polymeric tails. Next, the hybridization buffer was replaced with the digest buffer, and the beads were incubated with *DNase I* to test for enzymatic digestion. Flow cytometry was performed at various time intervals (15 minutes, 1 hour, and 24 hours) to record the effect of the nuclease on duplex number over time by monitoring the changes in fluorescence intensity as labeled, hybridized targets were cleaved and released from the particle surface. Previously hybridized samples were split into three distinct 33.3 µL aliquots to allow for a positive, control and digest sample. The positive sample contained hybridized beads resuspended to 100 µL PBS 0.02% Tween and stored in the refrigerator. The control and digest samples were centrifuged for 2 minutes at 9.9kG and washed once in a 1X solution of NE Buffer for *DNase I*. The control and digest samples were then resuspended

samples to 50 µL NEBuffer for *DNase I*. Next, a concentration of 8 U/mL of DNase I was added to the digest sample only. In this case, 2 µL of the DNase I enzyme was added. The digest samples were gently inverted to mix. The control and digest samples were incubated at 37°C in the hybridization oven for 24 hours. Aliquot of 10 µL were removed from the digest samples at 10 minute, 1 hour and 24 hour time intervals. Each aliquot was washed 3X in 100 µL PBS with 0.02% and resuspended to 100 µL PBS with 0.02% Tween. A sample containing hybridized beads at time zero serves as a positive control. The second control is a sample containing hybridized beads with the NEB buffer added and incubated at 37.5 °C. Lastly, the digest samples contain DNA-functionalized beads incubated with *DNase I* at 37.5 °C. The target strands with longer tails, namely 30PEG and Ultralong Triblock, were expected to be more effective in sterically hindering nuclease activity.

### *DNAse I* Digest Kinetics of LNA Target Sequences

The final set of experiments involved covalently coupling single-stranded L20 probes to functionalize the surface of the 1 µm carboxylated polystyrene microspheres. Target strands of FITC labeled B9 ET LNA were hybridized to the coupled beads using procedures detailed in previous sections. The suspension volume of the entire experiment was increased to allow for room temperature and 37°C controls at every time point. For primary hybridization, 9 µL L20-functionalized beads were added to 91 µL PBS 0.02% Tween to prepare a ssuspension at 1% loading. Next, 300 µL FAM-labeled B9 ET LNA at 10.0 µM was added to the 1% bead suspension and vortexed. The samples were incubated for 24 hours at room temperature on rota-mixer. They were then vortexed and

centrifuged for 4 minutes at 9.9kG before removing the supernatant. This wash procedure was repeated 3X in 100 μL PBS 0.02% Tween. The hybridized samples were then resuspended in 300 μL PBS 0.02% Tween.

To set up the digest experiment, the 300 μL samples were split into three 90 μL aliquots in three tubes to allow for a room temperature control, a 37°C control and a digest sample. All samples were washed two times in 90 μL NE Buffer for *DNase I* by centrifuging for 4 minutes at 9.9kG during eash wash. The samples were resuspend in 90 μL DNase buffer. Once the buffer exchange was complete, 20 μL of solution was removed from the room temperature control and pipette into another tube containing 79 μL PBS with 0.02% Tween and 1 μL 0.5M EDTA to quench the reaction. This "t=0" sample was vortexed, centrifuged, washed 2X in 100 μL PBS with 0.02% Tween and resuspended in 100 μL PBS with 0.02% Tween.

To begin the digest, a concentration of 8U/mL, in this case 3 μL, of *DNase I* enzyme was added to the digest samples only and vortexed to mix. to the Digest sample and vortex to mix. The 37°C control and digest samples were incubated at 37°C in the hybridization oven. The room temperature control samples were incubated at room temperature on the rota-mixer. At times 10 minutes, 1 hour and 24 hours, 20 μL of solution was removed from each sample and pippetted into pre-labeled tubes containing 79 μL PBS with 0.02% Tween and 1 μL 0.5 M EDTA. These samples were vortexed, centrifuged, washed 2X in 100 μL PBS with 0.02% Tween and resuspended in 100 μL PBS with 0.02% Tween. The

time-dependent nuclease activity was monitored using flow cytometry to determine the number of labeled duplexes remaining following incubation with *DNase I*.

# CHAPTER 3

# RESULTS

## Hybridization of Primary Targets without Polymeric Tails

The surface density of primary target as a function of base pair matches was measured using flow cytometry. This initial experiment investigated the effects of base pair length on hybridization ability showed that maximum duplex formation occurs for 14 base pair length of DNA as shown in Figure 3. Thus, the 14 base-long targets were chosen for all subsequent studies involving polymeric tails. These results were consistent with those conducted by Chris Tison in a previous study [5].

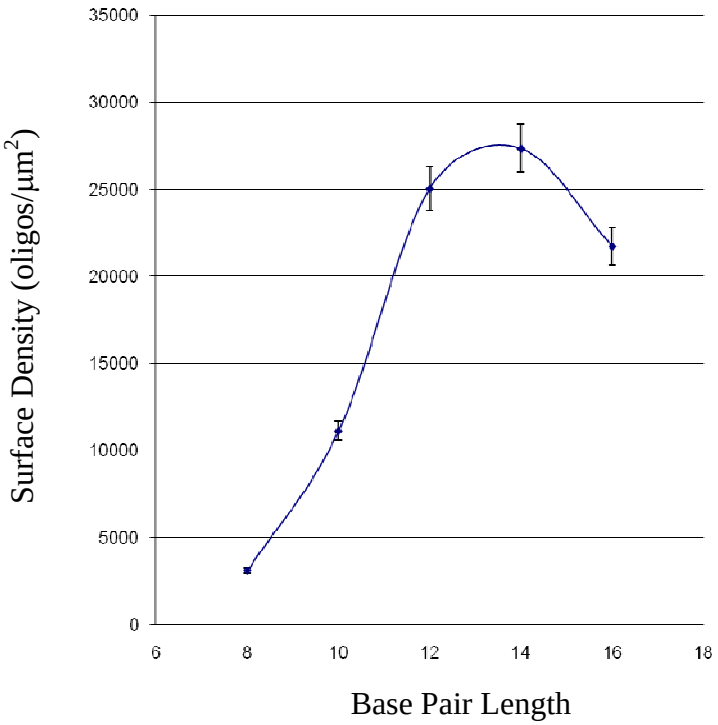


**Figure 3.** Comparison of hybridization activity of target with varying base-pair (BP) matches to probe strands.

**Hybridization of Primary Targets with Polymeric Tails**

After confirming that maximum hybridization occurs with 14 base pair long strands of DNA, we measured the surface duplex density using 14 base-long target strands with tails of various lengths and chemistries as shown in Figure 4.

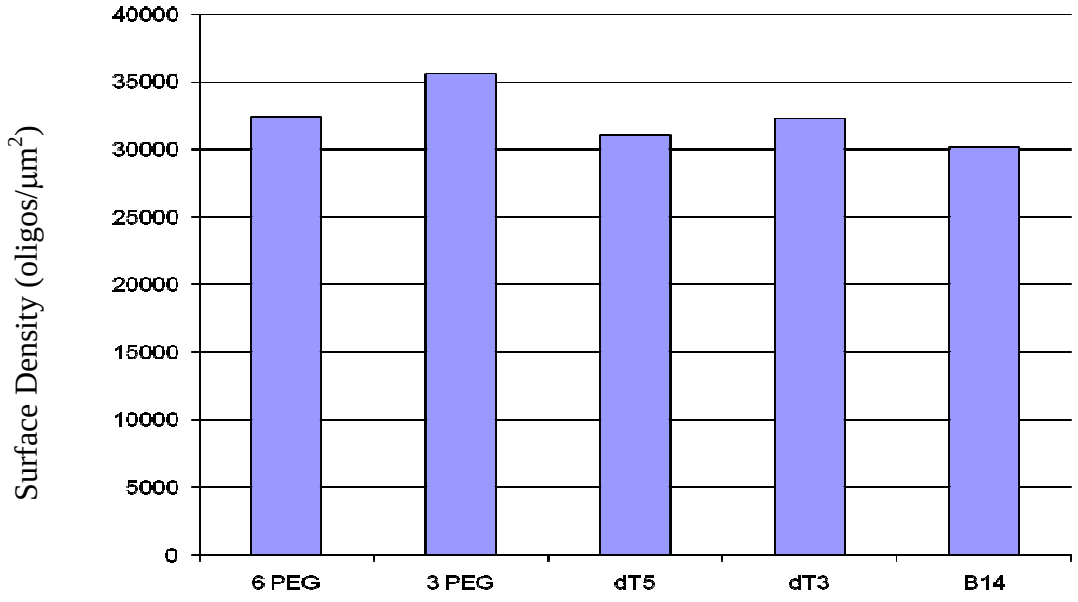


**Figure 4.** Surface duplex density values for 14 base-long targets with various polymeric tail lengths and chemistry. Only small differences in duplex densities were found to exist between PEG and Thymine-based polymeric tails of varying lengths or molecular weights.

**DNAse Digest Kinetics of DNA with Polymeric Tails**

The next objective of this research is to determine whether tails with different chemistries and lengths have varying abilities to inhibit nuclease activity over time. We expected that

target strands with longer tails would be more effective in sterically hindering nuclease activity.

There were three controls in this experiment. The positive sample contained 1 μm polystyrene beads with hybridized targets and suspended in the PBS Tween buffer solution. This “positive” sample showed the initial hybridization events before the enzyme digests the samples. The “control” sample also contains 1 μm polystyrene beads with hybridized primary targets. It, however, was washed and suspended in NE Buffer for DNase I. This buffer was used to prepare the samples to the enzymatic digest and facilitated the activity of the enzyme. All digest samples were suspended in NE buffer for DNase I and left in the hybridization for the respective time points. The last control is the B14 sample. The B14 sample is a 14 BP long strand of DNA without a polymeric tail. It serves as a comparison to the samples with 14BP with polymeric tails that are expected to provide steric protection to enzymatic activity.

To investigate duplex stability and the effect of nuclease activity on the PEG and thymine tails, we introduced the enzyme *DNase I* and measured the surface density of target strands remaining over time as shown in Figure 5. Exhibiting a high surface density of remaining duplexes correlates to high stability of the primary target in the presence of nucleases. Since the duplex densities did, however, decrease significantly in the presence of nucleases, we concluded that the addition of any polymeric tail did not significantly hinder nuclease activity.

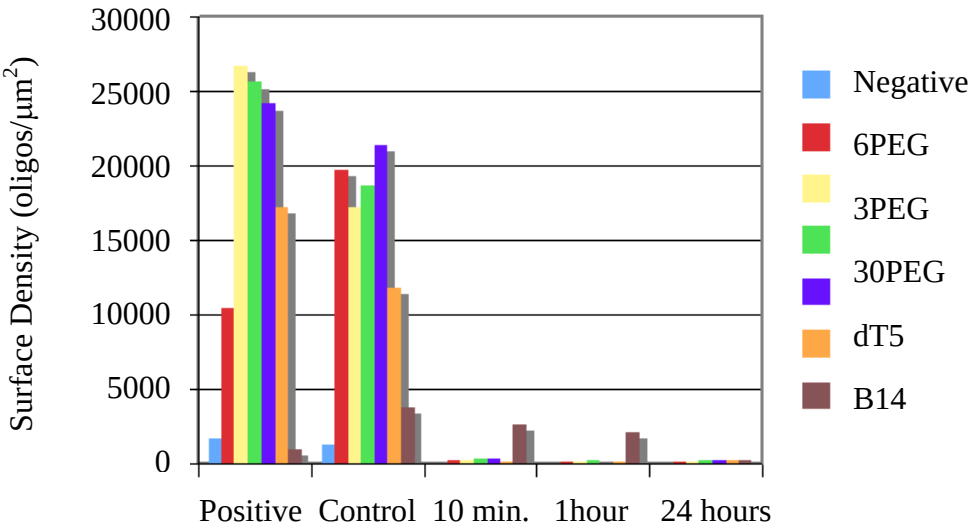


**Figure 5.** Average duplex density on microspheres following addition of 8 units/mL DNase as a function of inucubation times for various targets shown.

**DNAse Digest Kinetics of LNA**

As an alternative to DNA alone, we then investigated probe and/or target strands comprised of ~30% LNA residues. Figure 6 shows the enzymatic digest of B9 ET LNA at 10 minute, 1 hour and 24 hour time intervals. The two controls in this study were hybridized beads at room temperature and hybridized beads at 37°C with no added enzyme. These controls were used to monitor thermal dissociation of the LNA target from the probe.

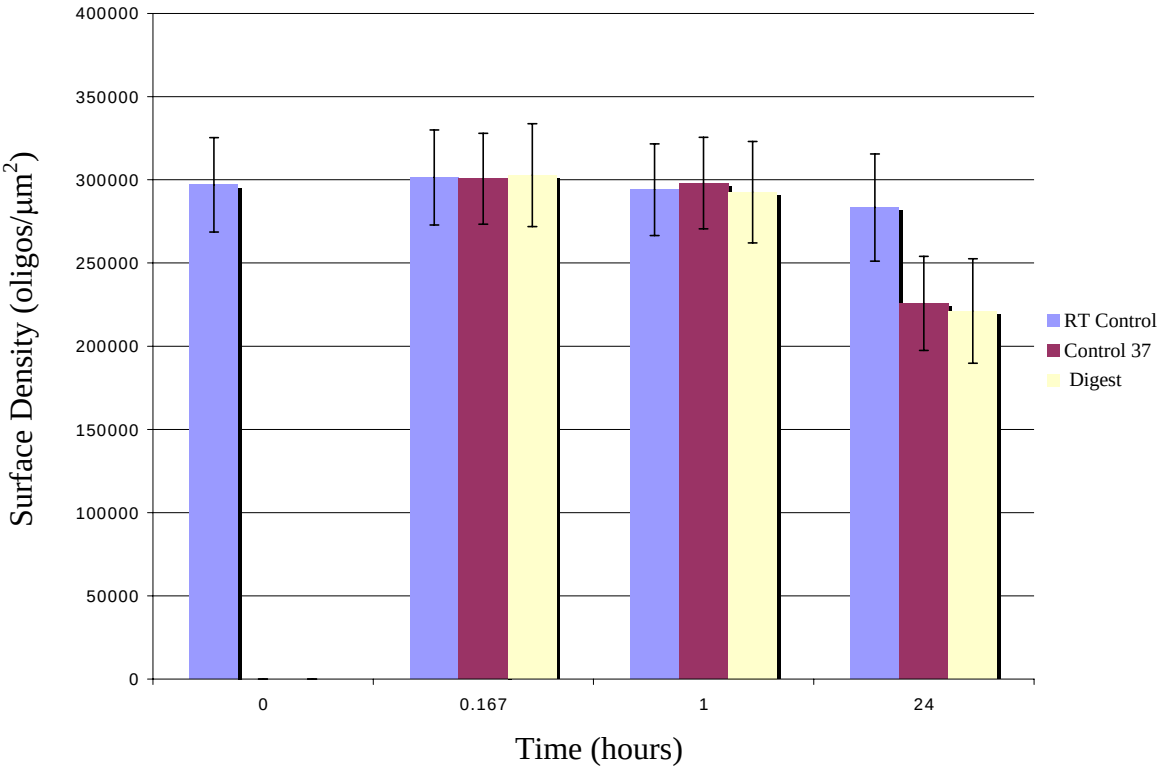


**Figure 6.** Average duplex density on microsphere as a function of incubation time with (Digest) and without (RT control, Control 37°C) nucleases present.

Since the duplex density does not decrease significantly over time, we concluded the LNA-based duplexes remain stable. At 24 hours, the digest sample is almost equivalent to the 37°C control. Here, the decrease in duplex density for this control may be a result of thermal dissociation and not digestion.

# CHAPTER 4

# DISCUSSION

## The Effect of Tail Length and Chemistry on Primary Hybridization

The surface duplex density using 14 base pair long target strands with tails of various lengths and chemistries were measured and compared. This initial study investigated the effects of tail chemistry as well as the effect of tail length on the ability to form duplexes or hybridize to the probe strand. The target strands with longer tails attached be expected to be less likely to hybridize efficiently due to steric interference with duplex formation. Only small differences, however, in duplex densities were found to exist between PEG and Thymine-based polymeric tails of varying lengths or molecular weights. Once hybridized, the target strands with longer tails attached were expected to be more effective in sterically hindering nuclease activity on the immobilized DNA duplexes.

## The Effect of *DNase I* on DNA Duplex Stability

This study investigated the effects of sterically protecting DNA duplexes from nuclease activity by including a polymeric "tail" on oligonucleotide targets. The target strands with longer tails attached were expected to be more effective in sterically hindering nuclease activity on the immobilized DNA duplexes.

Surprisingly, the addition various tails to the primary targets did not significantly hinder nuclease activity as shown in Figure 5. In fact, comparable digest activity was observed between the targets with polymeric tails and the targets with no polymeric tails (B14).

These results indicate that neither the short nor long tail lengths have appreciable effects on the hindrance of nuclease activity.

One concern with the initial design of the DNA digest experiment is the lack of extensive controls. The “positive” sample was suspended in PBS Tween buffer and stored in the refrigerator. It was intended to show the initial hybridization of primary targets before enzymatic digestion. However, because it was suspended in PBS Tween and was not incubated in the hybridization oven, the “positive” sample takes neither the effects of the buffer exchange with NE Buffer for *DNase I* nor the effects of thermal dissociation at body temperature into account. The “control” sample, suspended in the NE Buffer for *DNase I*, was incubated in the hybridization oven at 37°C for 24 hours. However, time points at 0 minutes, 10 minutes, and 1 hour were not examined for the control sample. Thus, the control sample may only be directly compared to the hybridization events of the 24 hour diget sample. This problem was addressed in the LNA digest experiments that followed. The controls for the LNA digests were redesigned such that the buffer exchange, thermal dissociation, and all time points were accounted for.

Another concern with the initial design of the digest experiments is the enzyme concentration. The digest samples were run at a relatively high concentration of *DNase I* at 8 units/mL *DNase I* comparable to concentrations in serum. Recently, we have observed that digests performed at 1.0 units/mL of *DNase I* (the approximate serum concentration found *in-vivo*) resulted in significantly slower (though still significant) clipping. For consistency, the *DNase I* digest experiments on LNA used the same enzyme

concentration of 8 units/mL. For future digest experiments with *DNase I,* a lower concentration of 1 unit/mL may be used to mirror the approximate serum concentration found *in-vivo*.

Ideally, we would have liked to determine the minimum tail length needed to maintain stability of the DNA duplexes in the presence of nucleases based on the previous studies regarding primary hybridization and enzymatic digests with DNase I. Since the addition of polymeric tails did not hinder the enzymatic cleavage of primary duplexes by DNase I, we modified the system and explored using locked nucleic acids (LNA) as an alternative to DNA. Since LNA has been reported to be more stable in the presence of nucleases compared to DNA, this approach may provide the chemical stability lacking in our current experimental system.

**The Effect of *DNase I* on LNA Duplex Stability**

These experiments are similar to prior experiments exploring the effects of *DNase I* on DNA duplex stability. However, the volume of the entire experiment was increased to add controls and EDTA was added to the PBS Tween solution to quench the reaction at each time point. The DNA studies only had control samples taken after 24 hours in the hybridization oven at 37°C. To ensure that control samples could be taken at each time point including a zero time point, the volume of the experiment was scaled up 3X. Control samples with the NE Buffer for DNase I were incubated in the hybridization oven and aliquots were obtained at 0 minute, 10 minute, 1 hour and 24 hour time points. The concentration of the DNase I enzyme remained constant at 8 units/mL. The final

difference in handling is as follows: during the 1st wash at each time point, the samples were washed in 79 μL PBS Tween with 1 μL EDTA pH 8.0 to quench the reaction. This step ensured that the digest reaction was stopped exactly at each time point to allow time for the other washes.

Figure 6 shows the effect of DNase I on LNA duplex stability. The samples were digested with *DNase I* at a concentration of 8 units/mL, the same concentration as was used in the DNA digest studies. LNA demonstrated significant stability to enzymatic activity. After 24 hours, the digest and 37°C control yield the same remaining duplex density within allowable experimental variation. This result suggests that the hybridized primary targets modified with LNA are stable in the presence of nucleases and he dissociation that occurs may be attributed to thermal dissociation rather than digestion.

Thus while promising results have been found, the LNA digests need to be repeated to confirm stabilization results. We later discovered that the enzyme activity itself may have been diminished due to time.

# CHAPTER 5

# CONCLUSION

Results from the studies reported here provide key insights into translating the current model *in vivo* system into a practical drug delivery vehicle for individualized delivery. The tail lengths used in this study did not have appreciable effects on the hindrance of nuclease activity. The B14 target strands with various polymeric tails did not significantly hinder nuclease activity regardless of the length or chemistry of the polymeric tail. From DNA digest kinetics study, the use of PEG or thymine tails did not inhibit cleavage of DNA duplexes by nucleases. The LNA digest kinetics study demonstrated that using locked nucleic acids (LNA) as an alternative to DNA to provide the chemical stability lacking in our current experimental system is promising. Our results show DNA modified with LNA provide stability of the linkages in the presence of nucleases, even without polymeric tails present. Future digest experiments may use a lower concentration of *DNase I* since these samples were run at a relatively high concentration of *DNase I* as compared to concentrations in serum.

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# VITA

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